

OOGENESIS IN PINUS LARICIO.

WITH REMARKS ON FERTILIZATION AND EMBRYOLOGY.

CONTRIBUTIONS FROM THE HULL BOTANICAL
LABORATORY. XIV.

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(WITH PLATES IV-VI)

IN the autumn of 1896, while conducting laboratory work in the special morphology of the gymnosperms, I noticed puzzling peculiarities in the development of the nucleus of the oosphere, but the material lacked important stages and had not been properly fixed for cytological study. The next spring, however, at intervals of three or four days, ovules of *Pinus Laricio* Poir were collected which gave a fairly complete series from the separation of the neck cell from the central cell of the archegonium up to stages in which the embryos had been thrust through the base of the oospore by the elongating suspensors.

For the earlier stages up to the cutting off of the ventral canal cell, the pair of ovules was merely cut off from the scale and dropped into the killing fluid; for stages from this point to the fusion of the pronuclei, the female gametophyte (prothallium) was usually removed from the ovule to insure rapid killing and fixing. In a part of the material, however, the nucellus was retained to show the course of the pollen tube to the oosphere. In all the later stages the gametophyte was removed from the ovule. All material was fixed on the spot, nothing being taken from cones which had been removed from the tree for more than fifteen minutes. In some cases as many as twenty ovules were taken from a single cone and kept in a separate bottle. These showed almost an identical stage of development. This uniformity occurs also in the sporangia of staminate cones in which the pollen mother cells undergo division at nearly the same time, the sporangia at the base being only slightly more

advanced than those at the tip. In the ovulate cone, at the time of the division of the mother cell into potential megaspores, the sporangia at the base are slightly more advanced, but they do not develop much further. For stages figured in this paper, the best material is found in the middle three fifths of the cone.

In my material, all of which was collected in the vicinity of Chicago, the ventral canal cell, in the season of 1897, was cut off about June 21, and fusion of the pronuclei occurred about a week later. In the season of 1896, all stages appeared more than two weeks earlier.

Various fluids were used for killing and fixing. For mitotic phenomena the most satisfactory results were secured by allowing Flemming's weaker solution to act for about two hours and then replacing it by a chrom-acetic solution (0.75^{gm} chromic acid and 0.25^{cc} to 100^{cc} water) in which the material remained for one or two days. In this way the advantages of the Flemming's solution seem to be secured without the objectionable blackening. The chrom-acetic acid used alone gave excellent results. Carnoy's fluid (absolute alcohol 6 parts, chloroform 3 parts, acetic acid 1 part), followed by cyanin and erythrosin, is good for tracing nuclei in the pollen tube and for differentiating the granules and network within the nucleus. Corrosive sublimate-acetic acid could hardly be recommended for achromatic structures in Pinus. Hermann's fluid and Merkel's fluid failed to give as good results as the much less expensive chrom-acetic acid. The popular safranin gentian-violet orange combination gave the most definite stain for achromatic structures—but Haidenhain's iron-alum-haematoxylin used alone, or followed by acid fuchsin, was better for most stages in the development of the oosphere nucleus.

THE VENTRAL CANAL CELL.

No attempt was made to secure a complete series of early stages leading up to the condition represented in *fig. 1*, which shows the nucleus of the central cell just before the division separating the ventral canal cell from the oosphere. At this stage there is often only a single nucleolus, and very seldom

more than two or three. This is worth noting, for the oosphere nucleus, soon after the cutting off of the ventral canal cell, contains a very large number of nucleoli. The chromatin is extremely scanty, unless, perhaps, it is in a diffuse state or forms a part of the nucleoli.

The mitotic figure concerned in the cutting off of the ventral canal cell presents a very characteristic appearance. The spindle is strongly developed and the part lying in the oosphere is sometimes quite convex, while the part within the ventral canal cell is concave (*fig. 2.*) The ventral canal cell usually begins to show signs of disorganization almost as soon as it is cut off. In this figure the nucleus had evidently reached the spirem stage before it began to disorganize. A similar difference between the nucleus of the ventral canal cell and that of the oosphere at this stage was noticed in several cases. In *fig. 3* the nucleus had developed further before disorganization set in, for a fully formed nuclear membrane is present and the chromatin is in the form of nucleoli or irregular masses. It is not unusual for the ventral canal cell to become separated from the egg as shown in this figure. In such cases the separation is due to the splitting of the wall between the two cells, and in some cases, even when no separation has taken place, as in *figs. 6* and *7*, the wall can be seen to be double. A later stage is shown in *fig. 5*, in which the ventral canal cell is reduced to a deeply staining mass, probably of mucus-like consistency, in which no structure is discernible. *Fig. 4* represents a ventral canal cell of about the same age as the preceding, but the chromatin is not so abundant and the other cell contents are scanty. It does not seem probable that such a cell, in its later stages, would present the appearance shown in *fig. 5* or exert such an influence upon the course of the pollen tube as might be expected in the latter case. In both *fig. 5* and *fig. 6*, the nuclear membrane has entirely disappeared, and no structure can be made out except that the mass is more or less homogeneous and contains a few nucleoli. That the nucleoli here represent the chromatin is proved by a very complete series, of which only a few stages (*figs. 2, 3* and *5*) are figured.

In later stages it may be difficult to identify the ventral canal cell as a whole, but vestiges of it in one form or another can usually be found up to the time when the pollen tube enters the neck of the archegonium, and occasionally traces may be seen even after the sporophyte is considerably advanced (*fig. 19a, v.*).

While the above account gives the usual history of the ventral canal cell, there are other rather exceptional cases which belong to an opposite course of development and are interesting on account of their bearing upon the homology of the ventral canal cell. *Fig. 7*, which is drawn to the same scale as *fig. 2*, shows an enormous spindle, somewhat loosely attached to the daughter nuclei. This ventral canal cell is eight or ten times as large as the typical one shown in *fig. 2*. It contains several proteid vacuoles like those of the oosphere, and its nucleus is undergoing the usual developmental changes which will be described for the nucleus of the oosphere. In this particular case, the nuclei of the oosphere and ventral canal cell are of nearly the same size and are in the same stage of development. If a pollen tube should enter, it seems reasonable to suppose that fertilization of the ventral canal cell might result. A later stage in such a course of development is represented in *fig. 10*, in which the nucleus has almost reached the condition presented by an oosphere nucleus just before conjugation. In *fig. 8* the nuclei are in about the same stage as in *fig. 7*, but the wall between the ventral canal cell and the oosphere has broken down, leaving both nuclei free in the oosphere. Fragments of the immense spindle are scattered throughout the oosphere. In some of these a strong line represents a part of the cell plate; in others the fibers are drawn into wisps resembling tips of bipolar spindles. A similar case is shown in *fig. 9*, but here the nuclei have nearly reached the size and stage of development shown in *fig. 17*. Still another example is shown in outline in *fig. 19a, v.* In *figs. 7-10*, the nucleus of the ventral canal cell, on account of its more favorable position, would be more likely to secure fertilization than the more remote nucleus of the oosphere. One might

suggest that a stage like *fig. 9* represents the two pronuclei within the egg, or that fertilization has already taken place and the two nuclei are the first two nuclei of the sporophyte. While the series shown in *figs. 7, 8, and 9* affords an answer to such suggestions, it may be added that vestiges of the pollen tube can almost always be detected above the neck of the archegonium until the oospore nucleus has divided two or three times. No evidences of fertilization were visible in these cases, and, besides, the upper nucleus is far too large for a male pronucleus just escaped from a pollen tube. If fertilization has not taken place, the second supposition is already excluded; but it may be added that the nuclei are too large for the first nuclei of the sporophyte, they lack the very characteristic appearance of the latter, and, further, their position is against any such interpretation.

The origin of the ventral cell has led several writers to regard it as the homologue of the egg. That this homology is correct, the series just described furnishes practically complete proof as far as origin and function can furnish it, the only thing lacking being an actual case of fertilization.

THE DEVELOPMENT OF THE OOSPHERE NUCLEUS.

The development of the nucleus of the oosphere differs decidedly from the usual course of development ascribed to the nucleus of the angiosperm oosphere. After the cutting off of the ventral canal cell, the nucleus of the oosphere increases enormously in size, as will be readily observed by comparing *figs. 2 and 17*.¹ During this increase in size, there takes place in the nucleus a series of changes which for some time it was difficult to interpret. In *fig. 2* the nucleus of the oosphere does not differ much in size or in any other perceptible respect from the nuclei in the sheath about the oosphere. In this figure the nucleus is in a typical spirem stage. The numerous and often coarse radiations about the nucleus were not observed in similar

¹ All the figures are drawn to the same scale except 14 and 15, and the outline sketches, 9, 19a, 24a, 25a, 26a, and 27a.

stages of prothallial cells. From this point the development is so extremely rapid that, in over three hundred preparations, less than a dozen nuclei showed stages intermediate between *figs. 2* and *11*. One of these is shown in *fig. 6, n. o.* (The proteid vacuoles, *p*, bear a striking superficial resemblance to nuclei.) In this nucleus nearly all the chromatin is in the form of nucleoli. A few linin threads are present, and associated with them is a very small quantity of chromatin. In other nuclei of about the same age, *figs. 7, 8*, no linin is visible, and all the chromatin is in the form of nucleoli. The greater number of these nucleoli stain blue with cyanin and erythrosin, but some stain red. The mere size of the nucleoli has no influence upon these reactions. I have not felt it safe to rely upon staining reactions for identifying chromatin, but have depended upon a close morphological series leading up to undoubted chromosomes. After all trace of the original linin network has disappeared and all the chromatin is in the form of nucleoli, another rather peculiar linin network appears. This and all the nucleoli stain intensely with iron-alum-haematoxylin. At first sight it would seem that we are dealing with a chromatin network, but if the second application of iron-alum be sufficiently prolonged, only a few of the nucleoli remain black, the stain being completely extracted from the network and from the rest of the nucleoli (*fig. 11*). If acid fuchsin be added to such a preparation, the network and the decolorized nucleoli stain red. A later stage, in which the decolorizing has not been carried so far, is shown in *fig. 12*. Details from nuclei in slightly later stages than *fig. 12* are shown in *figs. 14* and *15*, in which nearly all of the nucleoli are arranged upon the linin threads. Soon after this stage, the network begins to resolve itself into a granular substance, thus leaving the nucleoli scattered irregularly throughout the nucleus. Some of the nucleoli contribute to the granular substance, while others migrate from all directions toward a certain point, usually at the center, or a little above the center, of the nucleus. In *fig. 16* the network, while still distinguishable, has begun to break up, and nearly all of those nucleoli which

stain black with iron-alum-haematoxylin have taken a characteristic position. Some, however, which do not retain the stain, are scattered through the nucleus. *Fig. 17* shows a somewhat later stage. Only traces of the network are now visible; the granular condition is becoming quite pronounced and the nucleoli are arranging themselves in the form of a ring. The structure within this ring I was not able to make out definitely, but in most cases it seemed to be finely granular, with traces of threads more or less defined.

In *fig. 18* every vestige of the network has disappeared, and the nucleus is filled with an evenly granular substance. Nearly all the nucleoli have collected at the center. In *fig. 19* the groundwork of the nucleus shows a beautiful reticular structure, exactly like that of the cytoplasm outside the nucleus. The nucleoli are taking the form of elongated masses which represent definitely the chromatin of the nucleus. The threads seen in the central area are a part of the general reticulum.

The development of the male pronucleus was not studied in detail, but several stages, one of which is shown in *fig. 13*, indicate that the sequence is similar to that just described for the nucleus of the oosphere.

It seems probable that various bodies have been described under the term nucleolus. That some of these nucleoli represent chromatin has been proved both in animals and in plants. In animals the sequence described by Carnoy and Lebrun² for batrachians agrees more nearly with the conditions in *Pinus*. That the chromatin in the resting nucleus of *Spirogyra* is in the form of a nucleolus has been shown by several investigators, notably by Mitzkewitsch³. A chromatin nucleolus is also described by Davis⁴ for *Corallina*, one of the red algæ. A relation between chromatin and nucleoli has been noted in the

² La vésicle germinative et les globules polaires chez les batrachiens. *La Cellule* 14: 113-200. 1898.

³ Ueber die Kerntheilung bei *Spirogyra*. *Flora* 85: 81-124. 1898.

⁴ Kerntheilung in der Tetrasporenmutterzelle bei *Corallina officinalis* L. var. *mediterranea*. *Ber. d. deutsch. bot. Gesell* 16: 266-272. 1898.

higher plants, especially by Cavara⁵ and very recently by Duggar⁶.

Ikeno's⁷ figures and description of *Cycas revoluta*, while not dealing with details in the development of the nucleus of the oosphere, nevertheless indicate that the sequence is about as I have described it for *Pinus*. Blackman's recent work on *Pinus sylvestris* will be considered later. The staining reactions do not seem to furnish a reliable method of distinguishing chromatin nucleoli from other spherical bodies. In thin sections some nucleoli show a sharp differentiation into an outer deeply staining portion and an inner part which stains lightly (*fig. 29*). *Fig. 28* shows a peculiar case. There seemed to be a crack in the nucleolus, and upon applying a gentle pressure the central portion came out from the shell. It might be suggested that the presence or absence of this outer portion, or, when present, its relative thickness, may account in some measure for the reaction to stains. That the staining does not identify chromatin nucleoli is shown by the fact that many of the "nucleoli" in the proteid vacuoles stain intensely black with iron-alum-hæmatoxylin, blue with cyanin, or red with safranin.

FERTILIZATION.

Strasburger's⁸ figure of *Picea* shows conjugating nuclei of equal size. According to Coulter⁹, one of the male nuclei in *Pinus Laricio*, after its entrance into the oosphere, increases in size until, at the moment of fusion, it is as large as the female pronucleus. In Ikeno's figures of *Cycas*, and Blackman's¹⁰ of *Pinus*

⁵Intorno ad alcune strutture nucleari. Atti del R. Istituto Botanico dell'Università di Pavia 5:1-49. 1897.

⁶On the development of the pollen grain and embryo sac in *Bignonia venusta*. Bull. Torr. Bot. Club 26:89-105. 1899.

⁷Untersuchungen über der Geschlechtsorgane und den Vorgang der Befruchtung bei *Cycas revoluta*. Jahrb. f. wiss. Bot. 32:557-602. 1898.

⁸Befruchtung u. Zelltheilung. Leipzig. 1878.

⁹Notes on the fertilization and embryogeny of conifers. Bot. Gaz. 23:40-43. 1897.

¹⁰On the cytological features of fertilization and related phenomena in *Pinus sylvestris*. Phil Trans. Roy. Soc. of London. B. 190:395-426. 1898.

sylvestris, the male pronucleus is many times smaller than the female. In the present work, although the male pronucleus was often observed within the oosphere, no case was found in which the pronuclei were just coming into contact.

One fine preparation was obtained which shows the chromatin of the two pronuclei within the original limits of the female pronucleus (*fig. 20*). Both pronuclei are in the spirem stage, and show the chromatin granules evenly distributed upon a delicate linin thread. The kinoplasmic threads seem to be developing almost exclusively in the region of the male pronucleus, but it is hardly safe to draw any conclusions. A later stage, which I am not ready to interpret, is shown in *fig. 21*. The spirem thread has become perfectly smooth and has the same diameter as a chromosome in the anaphase of the first division of the fusion nucleus, but the segmentation into chromosomes is not yet complete. It seems possible that the upper group represents the male pronucleus, and the lower the female.

The first division after fertilization (*fig. 22*) shows a beautiful figure with a very strongly developed spindle, some of its kinoplasmic threads reaching from pole to pole while others merge insensibly into the surrounding groundwork of the nucleus. The chromosomes are very definitely U-shaped. After the second division has taken place and the four free nuclei have begun to move toward the base of the oospore, they show a characteristic tangential striation (*fig. 23*) which seems to be caused by a rotation of the nuclei as they descend. In the first division of these four nuclei, after they have reached the base of the oospore (*figs. 24, 24a*), the spindle is extremely broad and multipolar, but in later divisions (*figs. 26, 26a*) the spindle is of the usual bipolar type. This figure gives a typical view of the U-shaped chromosomes as they appear in these divisions, just before becoming separated from each other. Although several early phases in mitosis were found in the nuclei of the partially segmented portion of the oospore (*figs. 25, 25a*), no later stages were obtained. A fairly complete series was obtained showing mitosis in sporo-

phytes older than that represented in *fig. 26*. These show that the chromosomes do not resolve themselves into nucleoli, but pass into an ordinary reticulum.

A peculiar monstrosity was noted in one preparation (*figs. 27, 27a*). Instead of the usual free cell formation, each division of the nucleus has been followed by the formation of a wall, so that a somewhat spherical embryo has been formed near the center of the oosphere, about which no trace of a pollen tube could be found.

ARCHOPLASM AND CENTROSOMES.

After the large spindle concerned in the separation of the ventral canal cell from the oosphere has broken up, portions of it become scattered throughout the oosphere (*figs. 8, 19a*). One of these fragments (*19a, s.*) is shown in detail in *fig. 32*. Some of these fragments (*fig. 31, s*) in their later stages show a perfect transition from kinoplasmic fibers to the ordinary reticular structure of the oosphere. Such cases, together with those like *fig. 22*, make it probable that the kinoplasmic fibers do not arise from any specific kinoplasmic or archoplasmic substance but rather from a rearrangement of general reticulum, whether it be outside the nucleus or inside. Such a view, while not popular with botanists, has able supporters among zoölogists.

I was not able positively to identify centrosomes at any time during this work, but in several preparations clearly defined areas resembling the archoplasmic regions in *Ascaris* were observed near the male nuclei in the pollen tube (*fig. 13.*) The failure to find centrosomes has not convinced me that they are absent from the male nuclei.

The archoplasmic areas shown in *fig. 12* were observed in only one case, although nearly a hundred nuclei in approximately the same stage were examined. These areas, one inside the nucleus and the other outside, are sharply differentiated and are giving rise to rather coarse threads. This is not the usual method of spindle formation for this nucleus and may have nothing at all to do with the spindle.

BLACKMAN'S WORK.

After this paper had been practically completed, there appeared a very important work on *Pinus sylvestris* by Mr. Blackman (*l. c.*) Since this work has already been reviewed in the March number of this journal, only a brief comparison will be made here. I can confirm his statements that the pollen tube does not enter the oosphere; that the pronuclei at the time of fusion are not in the resting condition; that the history of the spindle fibers indicates that they result from a rearrangement of the ordinary cytoplasmic reticulum; and that the number of chromosomes is twelve in the pollen mother cell, in the nuclei of the sheath of the oosphere (*fig. 30*), and in other parts of the prothallium.

My second series (*figs. 7-10*), on the development of the ventral canal cell, was not observed by Blackman. His term maturation is not used, because it already has a definite and very different application. The term metaplastm is not used in the present paper because the network is regarded merely as a somewhat peculiar *linin* network. In the development of the nucleus of the oosphere, he neither figures nor describes the chromatin history in detail, but the points mentioned do not indicate the sequence described in this paper. He finds the chromosomes of the first segmentation nucleus V-shaped; *fig. 22* of this paper shows them U-shaped. He figures parallel threads around the four segmentation nuclei; although this appearance was occasionally noted in my preparations, the fibers usually had the arrangement shown in *fig. 23*. On the whole, it must be said that, while the two papers cover approximately the same ground, the more detailed work of each, having been done in different parts of the subject, is complementary. The general results, however, are to a great extent mutually confirmatory.

SUMMARY.

1. While the ventral canal cell usually disappears soon after it is formed, in some cases it persists and its nucleus becomes as large as that of the oosphere, passing through a similar develop-

mental history. New support is thus given to the theory that the ventral canal cell is the homologue of the egg.

2. In the development of the oosphere nucleus the chromatin takes the form of nucleoli which finally collect from all parts of the nucleus to a definite area near the center and there develop into a typical spirem. The linin often stains like chromatin.

3. After the male pronucleus is within the oosphere nucleus, the chromatin of the two pronuclei appears as two distinct masses in the spirem stage. Perhaps segmentation of the two spirems occurs while they are still separate.

4. Although centrosomes were not positively identified in any part of the work, appearances favor the supposition that they may accompany the male nuclei.

5. The fate of the spindle indicates that the kinoplasmic fibers arise through a transformation of the cytoplasmic reticulum.

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EXPLANATION OF PLATES IV-VI.

A Zeiss 2^{mm} apochromatic homogeneous immersion objective and compensating ocular no. 4, with camera lucida, were used for all drawings except figs. 14 and 15 (comp. oc. no. 18) and the outline sketches 9, 19a, 24a, 25a, 26a, and 27a, which were made with low power dry lenses. All drawings are reduced one half in photo-engraving.

Abbreviations: *n. o.*, nucleus of oosphere; *n. v.*, nucleus of ventral canal cell; *p.*, proteid vacuole; *s.*, portion of spindle; *v.*, remains of ventral canal cell.

FIG. 1. Nucleus of central cell before cutting off the ventral canal cell.

FIG. 2. Cutting off of the ventral canal cell.

FIGS. 3, 4, 5. Three views of the ventral canal cell.

FIG. 6. Apex of the oosphere, showing nucleus of oosphere, proteid vacuoles and the ventral canal cell.

FIG. 7. Extremely large ventral canal cell and spindle connecting it with the nucleus of the oosphere.

FIG. 8. The wall between ventral canal cell and oosphere has broken down, leaving the nucleus of the ventral cell free in the oosphere. Fragments of the spindle scattered through the oosphere.

FIG. 9. Similar to the preceding, but more advanced.

FIG. 10. Ventral canal cell with nucleus developed nearly to the fertilization stage.

FIGS. 11, 12, 16-19. Stages in development of nucleus of the oosphere.

FIG. 13. Two male nuclei in end of pollen tube just as it enters the neck of the archegonium.

FIGS. 14, 15. Nucleoli arranged on linin network; details from nuclei slightly older than *fig. 12*.

FIG. 19*a*. Sketch in which *n. o.* shows position of *fig. 19* and *s* the position of *fig. 32*.

FIG. 20. Fertilization.

FIG. 21. Later stage in fertilization.

FIG. 22. First division of the nucleus of the oospore.

FIG. 23. One and part of another of the four segmentation nuclei showing felt-like covering, with tangential threads predominating.

FIGS. 24, 24*a*. First division of one of the four free nuclei after passing to the base of the oospore.

FIGS. 25, 25*a*. Spirem in nucleus in the partially segmented portion of the oospore.

FIGS. 26, 26*a*. Nuclear figure showing shape of the chromosomes.

FIG. 28. Nucleolus showing deeply staining outer and lightly staining inner portions.

FIG. 29. Inner portion of nucleolus pressed out from the outer "shell."

FIG. 30. Nucleus of one of the cells sheathing the oosphere, showing the twelve chromosomes.

FIG. 31. Fragments of the ventral canal cell spindle showing transition between spindle fibers and cytoplasmic reticulum.

FIG. 32. Fragment of spindle. See *fig. 19a, s*.